

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**



Bescheinigung

Certificate

Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten internationalen Patentanmeldung überein.

The attached documents are exact copies of the international patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet international spécifiée à la page suivante.

Den Haag, den
The Hague,
La Haye, le

10. 03. 2004

Der Präsident des Europäischen Patentamts
Im Auftrag
For the President of the European Patent Office
Le Président de l'Office européen des brevets
p.o.


NATHALIE KUIPER

Patentanmeldung Nr. PCT/EP 00/00643
Patent application no.
Demande de brevet n°



Anmeldung Nr.:
Application no.:
Demande n°:

PCT/EP 00/00643

Anmelder:
Applicant(s):
Demandeur(s):

1. Vlaams Interuniversitair instituut voor Biotechnologie VZW - Zwijnaarde, Belgium
2. CORNELIS, Sigrid - Gent, Belgium (US only)
3. BEYAERT, Rudi - Zingem, Belgium (US only)

Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:

Novel internal ribosome entry site, vector containing same and uses thereof

Anmeldetag:
Date of filing:
Date de dépôt:

26 January 2000 (26.01.2000)

In Anspruch genommene Priorität(en)
Priority(ies) claimed
Priorité(s) revendiquée(s)

Staat: EP
State:
Pays:

Tag: 26 January 1999
Date: (26.01.1999)

Aktenzeichen: 99200216.2
File no.
Numéro de dépôt:

Benennung von Vertragsstaaten : Siehe Formblatt PCT/RO/101 (beigefügt)
Designation of contracting states : See Form PCT/RO/101 (enclosed)
Désignation d'états contractants : Voir Formulaire PCT/RO/101 (ci-joint)

Bemerkungen:
Remarks:
Remarques:

2/4

V1-002-V036

PCT REQUEST

Original (for SUBMISSION) - printed on 26.01.2000 10:26:42 AM

III-2	Applicant and/or inventor	applicant and inventor
III-2-1	This person is:	US only
III-2-2	Applicant for	BEYAERT, Rudi
III-2-4	Name (LAST, First)	Gentsesteenweg 16
III-2-5	Address:	B-9750 Zingem Belgium
III-2-6	State of nationality	BE
III-2-7	State of residence	BE
IV-1	Agent or common representative; or address for correspondence The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	common representative
IV-1-1	Name	VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE VZW
IV-1-2	Address:	Rijvisschestraat 120 B-9052 Zwijnaarde Belgium
IV-1-3	Telephone No.	+32 9 244 66 11
IV-1-4	Facsimile No.	+ 32 9 244 66 10
V	Designation of States	
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AP: GH GM KE LS MW SD SL SZ TZ UG ZW and any other State which is a Contracting State of the Harare Protocol and of the PCT EA: AM AZ BY KG KZ MD RU TJ TM and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT EP: AT BE CH&LI CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE and any other State which is a Contracting State of the European Patent Convention and of the PCT OA: BF BJ CF CG CI CM GA GN GW ML MR NE SN TD TG and any other State which is a member State of OAPI and a Contracting State of the PCT
V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AE AL AM AT AU AZ BA BB BG BR BY CA CH&LI CN CR CU CZ DE DK DM EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX NO NZ PL PT RO RU SD SE SG SI SK SL TJ TM TR TT TZ UA UG US UZ VN YU ZA ZW

OAV1 / RUB / IRES / 002-V036 / WO**Novel internal ribosome entry site, vector containing same
and uses thereof**

5

Field of the invention

The present invention relates to an isolated and/or recombinant nucleic acid molecule, preferably a cloned DNA sequence element, that can be incorporated into expression vectors for improving translation of a given mRNA and to enable
10 said translation of the mRNA in a cap-dependent but also, more importantly, in a cap-independent manner in eukaryotic cells. The DNA sequence element comprises the so-called internal ribosomal entry site, abbreviated as and called hereafter IRES.

15

Summary of the invention

It has been found by the current invention that two isoforms, p110 and p58 of PITSLRE protein kinase, can be translated from the same PITSLRE by an internal ribosome entry process. This means that p110 and p58, two proteins with putative
20 different functions, are translated from a single mRNA species by using two AUGs within the same reading frame. These two proteins share the 439 C-terminal amino acids that contain the kinase domain. The IRES in the polycistronic PITSLRE is the first IRES completely localized in the coding region of a cellular mRNA. Moreover, it was surprisingly found that the IRES element is cell cycle regulated. Translation of p58 occurs in the G2/M stage of the cell cycle.

25

Background of the invention

The PITSLRE protein kinase family is a large supergene family related to the master mitotic protein kinase, p34cdc2. PITSLRE protein kinases are encoded by the duplicated genes cell division cycle 2-like 1 (Cdc2L1) and Cdc2L2, which span

approximately 140 kb on human chromosome 1p36.3 (Gururajan *et al.*, 1998). These genes express almost identical protein kinases of 110 kDa, which contain at their C-terminal end the open reading frame of a smaller isoform of 58 kDa: p58PITSLRE. At present the function of PITSLRE kinases remains elusive. The
5 reason for the high number of different isoforms is also not clear. Only the p110 and p90 isoforms contain a 30-amino acid region comprised primarily of glutamic acid (83 %) (Xiang *et al.*, 1994). Shorter glutamic acid sequences can be found in all isoforms immediately following the first translational start site for the β 1 isoform. Several highly basic regions, which could function as bipartite nuclear
10 localization sequences, are found only in the p110 isoforms (Xiang *et al.*, 1994). The yeast two-hybrid system revealed a direct interaction between the RNA-binding protein, hRNPS1, and the p110 isoforms but not with the smaller isoforms (Loyer *et al.*, 1998).

The wide variation in the expression patterns of the different isoforms and their
15 levels of expression points to isoform specific functions. While p110 isoforms are ubiquitously expressed in asynchronous cell populations, overexpression of p58 in eukaryotic cells leads to a late mitototic delay due to an apparent failure of cytokinesis. The cells are sequestered at late telophase for an extended period of time (Bunnell *et al.*, 1990). The rate of cell growth in these cells is greatly
20 diminished. Conversely, diminished p58 mRNA levels in CHO fibroblasts are associated with enhanced cell growth, as measured by increased rates of DNA replication (Meyerson *et al.*, 1992). These observations suggest that p58 might participate in normal regulation of the cell cycle or cell death. Additionally, the chromosome region 1p36.3 is often deleted in neuroblastoma and many other
25 tumors. Deletion of this chromosome region occurs late in oncogenesis and is correlated with aggressive tumor growth, suggesting that one or more tumor suppressor genes may reside here (Eipers *et al.*, 1991). Another observation pointing to a role for PITSLRE kinases during cell cycle progression is that during early embryogenesis in *Drosophila*, when exponential proliferation takes place, the

expression level of PITSLRE specific transcripts, encoding the p110 homologue, is maximal. At a later stage when cell proliferation is attenuated, PITSLRE expression drops dramatically (Sauer *et al.* 1996).

Polycistronic messengers as such occur frequently in prokaryotic systems. It has been a long-established misunderstanding that said polycistronic messengers did not occur in eukaryotic systems because of the presence of the so-called "cap" at the start of mRNA. Indeed, initiation of translation of the majority of eukaryotic cellular and viral mRNAs results from attachment of ribosomes to the m⁷ G cap at the 5'-end of the mRNA followed by linear scanning to the initiation codon.

10 However, initiation of translation of a smaller number of eukaryotic mRNAs is 5'-end- and cap-independent, and instead results from direct attachment of ribosomes to an internal ribosomal entry site (IRES) within the 5' non-translated region (5' NTR) of the mRNA.

IRES elements were first reported in picornaviral mRNAs which are naturally uncapped but nonetheless efficiently translated (Jang *et al.*, 1988, J.Virol., 62:2636-43). Generally IRES cannot be identified by sequence homology; known IRES have been identified and defined functionally (Mountford and Smith, 1995, TIG, 11(5): 179-184). It appears that the conformation of the IRES sequence enables the binding on the ribosome.

20 It would be useful to identify a sequence element that endows any desired gene with the ability to be efficiently translated and to be translated in a cap-independent manner in particular. Moreover, it would be of great advantage to isolate a sequence element that is cell cycle regulated. Furthermore, it would be extremely useful to have IRES sequence elements with a preferably high translational efficiency to use in expression vectors as well as in gene therapy vectors in order to

25 control mRNA translation and therefore protein synthesis.

Detailed description of the invention

The current invention relates among others to the feature that p58 is produced from the PITSLRE mRNA by a mechanism of internal initiation of translation during the G2/M stage of the cell cycle. An embodiment of this invention is disclosed hereafter.

5 An SV40 early promoter driven p110PITSLRE isoform ($\alpha 2$ -2) fused to an E-tag at its C-terminal end was constructed. This plasmid was stably transfected in the IL-3 dependent pro B-cell line Ba/F3. This system has the advantage that cells can be synchronized in the G1 stage by growth factor depletion. Subsequent stimulation
10 with IL-3 drives the cells simultaneously through further stages of the cell cycle. Via immunoblotting using anti-E-tag the expression pattern of the transfected PITSLRE construct was studied during cell cycle progression in the Ba/F3-p110PITSLRE transfectants. The p110 isoform was constitutively present during the different stages of the cell cycle. However, only during the G2/M stage a 58
15 kDa form was co-expressed (figure 1A). Cell cycle progression was followed by FACS analysis (figure 1B). To make sure that this expression pattern was not a clone dependent phenomenon several other clones were analysed. All displayed a similar pattern.

Also a minor band of about 100 to 105 kDa was detected. It has been described
20 previously that the p110 isoforms have two potential translational starts near the start of the 5'NTR region. They are separated by 174 bp and they are located in two adjacent exons. A possible mechanism however has not been suggested. Perhaps this alternative initiation of translation is a consequence of leaky ribosome scanning. The alternative AUG (at position 283) is located in a more favourable
25 region for initiation of translation compared to the first AUG (position 112) (table 1).

The origin of the p58 product was initially thought to be the result of a proteolytical proces. It has been suggested that glutamic acid regions present in the p110 isoform are potential points of specific proteolytical cleavage. Recently, a

possible role for a caspase processed PITSLRE isoform has been suggested by studies of Fas- and TNF-induced cell death. (Lahti *et al.*, 1995, Beyaert *et al.*, 1997, Tang *et al.*, 1998). Transfectants were incubated with the broad spectrum inhibitor of caspases, zVAD.fmk and the PITSLRE expression pattern was analysed. As figure 1 shows, zVAD.fmk did not affect G2/M specific expression of p58, excluding the possibility that p58 was generated by proteolytic processing of the p110 isoform by resident caspases.

The p110 isoform contains at its C-terminal end the ORF of isoform p58. Therefore the G2/M-specific p58 protein was probably translated using the internal AUG as an initiation codon. To verify the latter hypothesis, the internal methionine was mutated to alanine and the mutant cDNA was transfected into the Ba/F3 cell line. Interestingly, when compared with wild type (fig. 2A) this mutation completely knocked out the p58 expression in the G2/M stage of the cell cycle whereas p110 expression remained unchanged (fig. 2B).

The expression of cyclin B1 as a marker for the G2/M stage of the cell cycle was used as control. Cyclin B1 is synthesized during late S, maximally expressed during the G2/M and finally degraded during the anaphase (fig. 2C).

To rule out the possibility that we mutated a potential proteolytical cleavage site in p110PITSLRE capable of generating a 58 kDa product, we introduced in the p110PITSLRE cDNA a frame shift by deletion of two guanosine nucleotides at positions 926 and 927 upstream of the internal AUG (fsp110PITSLRE). This mutation led to a short open reading frame of 867 nucleotides (fig. 2D). Western blot analysis of stable Ba/F3 transfectants of fsp110PITSLRE revealed that the p58PITSLRE was still produced in G2/M in the absence of p110PITSLRE expression (fig 2D).

Since expression of the transfected gene was controlled by a constitutive early SV40 promoter, regulation of p58 expression at the transcriptional level was excluded.

6

Next, it was determined whether p58 and p110 were both derived from one mRNA. It is possible that p58 is translated from another, second, messenger derived from the initially transfected cDNA. This second messenger could be produced by cleavage of the transfected messenger by a specific ribonuclease or could be induced by a cryptic promotor element present in the upstream sequence. The exogenous mRNA pattern was followed during cell cycle progression by Northern blot analysis with an E-tag specific probe in the Ba/F3 transfectants. One single messenger of 2.4 kb in all stages of the cell cycle was found at a constant expression level. The 2.4 kb band was absent in non-transfected cells. No additional messenger in the G2/M-specific cell lysates was detected. This shows that the p110 (α 2-2) mRNA, encoding p110^{PITSLRE} (α 2-2), gives rise to a second PITSLRE protein kinase of 58 kDa.

Endogenous PITSLRE protein kinase expression during cell cycle progression was also examined. As shown in figure 3, analysis of the Ba/F3 parental cells during cell cycle progression revealed a similar pattern compared to the Ba/F3/p110^{PITSLRE} transfectants. The p110 isoform is permanent present during the different stages of the cell cycle. In accordance with the observations in the transfectants, a strongly enhanced expression of p58 was detected in the G2/M-stage.

We further investigated the cell cycle dependence of p58^{PITSLRE} expression by Northern blot analysis using a fragment corresponding to the 3' end of the coding region of mouse p110^{PITSLRE} (Malek and Desiderio, 1994) as a probe. Based on the high conservation in this region between the different human isoforms, we assumed this probe would recognize all murine PITSLRE transcripts. This analysis indicated a single transcript of approximately 3.2 kb in all phases of the cell cycle (fig 3C), which is in agreement with previously published observations in mouse tissues (Malek and Desiderio, 1994). No additional G2/M-specific transcript accounting for expression of the 58 kDa product was detectable in Ba/F3 cells,

suggesting that G2/M-specific expression of p58PITSLRE was regulated at a post-transcriptional level.

Taken together, the present invention thus concerns a nucleotide sequence enabling a cell cycle dependent initiation of translation of mRNA. More preferably, said sequence is an IRES sequence and even more preferably said cell cycle
5 dependency is a G2/M cell cycle dependency. More specifically, the present invention relates to an isolated and/or recombinant nucleic acid molecule, preferably DNA, encoding at least a functional part of an eukaryotic internal ribosomal entry site, which site in the mitotic PITSLRE protein kinase gene
10 comprises the sequence as depicted in SEQ.ID.NO.1 or a functional part thereof. The present invention also concerns a nucleic acid molecule comprising at least a part of the sequence as depicted in SEQ.ID.NO.1 or a sequence at least substantially homologous thereto.

In another embodiment the nucleic acid molecule according to the invention relates
15 to at least a part of the sequence as depicted in SEQ.ID.NO.1 or a sequence which hybridizes under conventional conditions to at least a part of said sequence or its complementary sequence.

Unexpectedly, it has been found that SEQ.ID.NO.4, being an overlapping sequence of SEQ.ID.NO.5 and SEQ.ID.NO.1, has corresponding IRES functionality
20 according to the current invention. Therefore part of the invention is an isolated and/or recombinant nucleic acid molecule, preferably DNA, comprising at least the sequence as depicted in SEQ.ID.NO. 4, but also an isolated and/or recombinant nucleic acid molecule, preferably DNA, comprising at least the sequence as depicted in SEQ.ID.NO. 5 and in addition thereto an isolated and/or recombinant
25 nucleic acid molecule, preferably DNA, comprising at least the sequence as depicted in SEQ.ID.NO. 6.

Even more surprisingly, a deletion in the IRES sequence of a sequence with SEQ.ID.NO.7 resulted in a loss of the IRES functionality, indicating that this sequence can play essential role in IRES functionality. Therefore, part of the invention is an

isolated and/or recombinant nucleic acid molecule, preferably DNA, comprising at least the sequence as depicted in SEQ.ID.NO.7.

The invention also relates to a chimeric gene comprising the following operably linked polynucleotides:

- 5 a) a nucleic acid molecule according to the invention, and
- b) one or more control sequences.

Part of the present invention is also a vector comprising at least said nucleic acid molecule or comprising said chimeric gene mentioned above and an eukaryotic host cell comprising said nucleic acid molecule or comprising the chimeric gene
10 according to the invention.

Said vector can be conveniently an expression vector containing at least a single promoter. A derived expression system comprising an eukaryotic host cell according to the invention forms part of the invention as well.

Another aspect of the invention is a method for cap-independent translation of
15 mRNA by including in an expression vector, a translation control element or analogues thereof having the nucleic acid molecule as set forth in SEQ.ID.NO.1 and/or SEQ.ID.NO.4.

The sequences of the invention can be used to induce a cell cycle dependent initiation of translation in eukaryotic cells. In particular said sequences can be used
20 wherein the sequence is a cell cycle dependent IRES sequence, more preferably a G2/M-dependent IRES sequence.

In addition said vector or any of the sequences according to the invention can be used for the preparation of a pharmaceutical composition for the treatment and/or prevention of a disease by gene therapy. In this regard, the present invention
25 relates to the use of any recombinant gene endowed with an IRES sequence giving it the ability to be specifically translated in cells that are in the G2/M phase of the cell cycle. Said constructs are therefore extremely useful in gene therapy approaches that target proliferating cells. In such cases, the IRES drives the translation of a mRNA encoding a protein that is toxic or growth inhibitory for the

cells in which it is expressed (e.g. RB, FAS ligand, thymidine kinase, caspases; reviewed in Tio *et al.*, 1998), or which restores the expression of proteins that are damaged or missing. The G2/M specific activity of the IRES results in specific expression of the protein in proliferating cells, leaving the other cells intact. One
5 embodiment of the invention is the use of the IRES in cancer gene therapy by blocking tumor cell growth or inducing tumor cell death. Additionally, blocking the growth of tumor blood vessels, which are required for the growth of tumors, can be achieved.

Another embodiment of this invention is the use of the IRES in the treatment of
10 restenosis, which results from the abnormal growth of blood vessel smooth muscle cells following angioplasty for coronary artery disease and peripheral vascular disease. A vessel-expansion technique called balloon angioplasty is one of the most popular treatments for the cardiovascular blockages that commonly lead to heart attacks. However, due to the mild tissue damage at the surgical site, many
15 patients experience an exaggerated, post-operative healing response, whereby vascular cells proliferate to form a scar that re-clogs the artery. This re-occlusion of the artery is called restenosis and affects up to 50% of the patients receiving primary balloon angioplasty (Schwartz *et al.*, 1992). The effectiveness of
20 adenovirus as a gene therapy vector in animal models of restenosis is well documented (Gerard and Collen, 1997). Therefore, recombinant adenoviral vectors in which the IRES drives the expression of a gene product which affects smooth muscle cell proliferation are constructed in order to inhibit smooth muscle cell proliferation in culture and in pig coronary artery balloon angioplasty model of restenosis. The IRES mediated gene therapy can be delivered locally in an
25 integrated angioplasty procedure using catheter-based gene delivery (Varenne *et al.*, 1999), or with other methods known to the people skilled in the art.

The present invention also relates to the use of IRES sequences to study the role of proteins in mitosis or to screen for novel regulators of cell proliferation. Said regulators can be any chemical or biological compound, including simple or

10

complex inorganic molecules, peptides, peptido-mimetics, proteins, antibodies, carbohydrates, nucleic acids or derivatives thereof. A change in expression of the reporter gene indicates a role for the putative regulator in cell cycle regulation. In case said regulator is a protein, peptide, antibody or nucleic acid, it can be expressed in a bicistronic or polycistronic vector that carries the G2/M-specific IRES as an intercistronic sequence in front of the reporter gene cistron.

In order to clarify what is meant in this description by some terms a further explanation is hereby given:

10

The terms "gene(s)", "polynucleotide", "nucleic acid sequence", "nucleotide sequence", "sequence" or "nucleic acid molecule(s)" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA, and RNA. It also includes known types of modifications, for example, methylation, "caps" substitution of one or more of the naturally occurring nucleotides with an analog. "Recombinant nucleic acid molecule" as used herein refers to a polynucleotide of genomic, cDNA, semi synthetic or synthetic origin which, by virtue of its origin or manipulation is:

- linked to a polynucleotide other than that to which it is linked in nature, or
- does not occur in nature.

An "expression vector" is a construct that can be used to transform a selected host cell and provides for expression of a sequence or expression of a gene in the selected host. Expression vectors can for instance be cloning vectors, binary vectors or integrating vectors.

"Expression of a sequence" or "expression of a gene" is the transcription of said sequence or gene in RNA, and where applicable, the translation of the coding sequence into protein.

30 An "expressed sequence" is a nucleotide sequence which is transcribed into

mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences and consists of the sequence between the start of transcription and the stop of transcription. The translated sequence is called coding sequence. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. An expressed sequence can include, but is not limited to mRNA, cDNA, recombinant nucleotide sequences or genomic DNA, while introns may be present as well under certain circumstances.

"Expression of a protein" means the production of a protein or nucleotide sequence in the cell itself or in a cell-free system. It includes transcription into an RNA product, post-transcriptional modification and/or translation to a protein product or polypeptide from a DNA encoding that product, as well as possible post-translational modifications.

"Control sequence" refers to regulatory DNA sequences which are necessary to affect the expression of expressed sequences to which they are ligated and to sequences necessary for the translation of coding sequences. The nature of such control sequences differs depending upon the host organism. In prokaryotes, control sequences generally include promoter, ribosomal binding site, and terminators. In eukaryotes generally control sequences include promoters, terminators and, in some instances, enhancers, transactivators or transcription factors. The term "control sequence" is intended to include, at a minimum, all components the presence of which are necessary for expression and translation, and may also include additional advantageous components.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to an expressed sequence and/or coding sequence is ligated in such a way that expression of the expressed sequence and/or translation of the coding sequence is achieved under conditions compatible with the control sequences. In case the control sequence is a promoter, it is obvious for

a skilled person that double-stranded nucleic acid is used.

The terms "protein" and "polypeptide" used in this application are interchangeable.

"Polypeptide" refers to a polymer of amino acids (amino acid sequence) and does not refer to a specific length of the molecule. Thus peptides and oligopeptides are included within the definition of polypeptide. This term does also refer to or include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

"Fragment of a sequence" or "part of a sequence" means a truncated sequence of the original sequence referred to. The truncated sequence (nucleic acid or protein sequence) can vary widely in length; the minimum size being a sequence of sufficient size to provide a sequence with at least a comparable function and/or activity of the original sequence referred to, while the maximum size is not critical. In some applications, the maximum size usually is not substantially greater than that required to provide the desired activity and/or function(s) of the original sequence. Typically, the truncated amino acid sequence will range from about 5 to about 60 amino acids in length. More typically, however, the sequence will be a maximum of about 50 amino acids in length, preferably a maximum of about 30 amino acids. It is usually desirable to select sequences of at least about 10, 12 or 15 amino acids, up to a maximum of about 20 or 25 amino acids.

"Transformation" as used herein, refers to the transfer of an exogenous polynucleotide into a host cell, irrespective of the method used for the transfer. The polynucleotide may be transiently or stably introduced into the host cell and may be maintained non-integrated, for example, as a plasmid, or alternatively, may be integrated into the host genome. Many types of vectors can be used to transform cells. These transformation methods are known to the person skilled in the art.

"Functional part of" means that said part to which subject it relates has substantially the same activity as the subject itself, although the form, length or structure may vary.

The term "substantially homologous" refers to a subject, for instance a nucleic acid, which is at least 50% identical in sequence to the reference when the entire ORF (open reading frame) is compared, where the sequence identity is preferably at least 70%, more preferably at least 80%, still more preferably at least 85%, especially more than about 90%, most preferably 95% or greater, particularly 98% or greater. Thus, for example, a new nucleic acid isolate which is 80% identical to the reference is considered to be substantially homologous to the reference.

Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridisation experiment under, for instance, conventional or preferably stringent conditions as defined for that particular system.

Similarly, in a particular embodiment, two amino acid sequences, when properly aligned in a manner known to a skilled person, are "substantially homologous" when more than 40% of the amino acids are identical or similar, or when more preferably more than about 60 % and most preferably more than 69% of the amino acids are identical or similar (functionally identical).

"Sense strand" refers to the strand of a double-stranded DNA molecule that is homologous to a mRNA transcript thereof. The "anti-sense strand" contains an inverted sequence which is complementary to that of the "sense strand".

"Cell cycle" or "cell division" means the cyclic biochemical and structural events associated with growth and with division of cells, and in particular with the regulation of the replication of DNA and mitosis. The cycle is divided into periods called: G₀, Gap₁ (G₁), DNA synthesis (S), Gap₂ (G₂), and mitosis (M).

The present invention also relates to nucleic acid molecules hybridizing with the above-described nucleic acid molecules and differ in one or more positions in comparison with these as long as they encode a comparable protein. By

"hybridizing" it is meant that such nucleic acid molecules hybridize under conventional hybridization conditions, preferably under stringent conditions such as described by, e.g., Sambrook (Molecular Cloning; A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)).

5 An example of one such stringent hybridization condition is hybridization at 4XSSC at 65 °C, followed by a washing in 0.1XSSC at 65 °C for one hour. Alternatively, an exemplary stringent hybridization condition is in 50 % formamide, 4XSSC at 42 °C. The invention also relates to nucleic acid molecules the sequence of which differs from the nucleotide sequence of any of the above-

10 described nucleic acid molecules due to the degeneracy of the genetic code.

"Homology" further means that the respective nucleic acid molecules or encoded proteins are functionally and/or structurally equivalent. The nucleic acid molecules that are homologous to the nucleic acid molecules described above and that are derivatives of said nucleic acid molecules are, for example, variations of said

15 nucleic acid molecules which represent modifications having the same biological function, in particular encoding proteins with the same or substantially the same biological function. They may be naturally occurring variations, such as sequences from other varieties or species, or mutations. These mutations may occur naturally or may be obtained by mutagenesis techniques. The allelic variations may be

20 naturally occurring allelic variants as well as synthetically produced or genetically engineered variants.

The present invention also relates to "vectors", particularly plasmids, cosmids, viruses, bacteriophages and other vectors used conventionally in genetic engineering that contain a nucleic acid molecule according to the invention.

25 Methods which are well known to those skilled in the art can be used to construct various plasmids and vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively,

15

the nucleic acid molecules and vectors of the invention can be reconstituted into liposomes for delivery to target cells.

5 BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Cell cycle dependent expression of transfected p58^{PITSLRE}.

(A) Western blot analysis with anti-E-tag antibodies of synchronized Ba/F3-p110PITSLRE cells grown in presence (zVAD⁺) or absence (zVAD⁻) of 100µmolar zVAD-fmk prepared at different time points after IL-3-stimulation. Corresponding cell cycle phases are indicated. (B) The percentage of cells that are in a specific phase of the cell cycle was determined by FACS analysis of the DNA content after staining with propidium iodide (diamond: G1; square: S; triangle: G2/M) FACS analysis was carried out as follows: Ba/F3 cells were IL-3 depleted for 14h to arrest cells in G1. DNA content was measured by freezing the cells in the presence of propidium iodide, and subsequent FACS analysis. Results are shown for a representative cell clone.

Figure 2: p58^{PITSLRE} is expressed in G2/M by internal initiation of translation on the full-length p110^{PITSLRE} mRNA.

Western blot analysis with anti-E-tag antibodies (A, B, D) or anti-cyclin B1 antibodies (C) of Ba/F3-p110PITSLRE cells (A), Ba/F3-mut-p110PITSLRE cells (B,C) or Ba/F3-fsp-110PITSLRE cells (D) prepared at different time points during cell cycle progression. Cells were synchronized in G1 by IL-3-depletion and released from this G1-block by subsequent stimulation with IL-3 for the times indicated. In (D) "c" is indicating the control experiment with Ba/F3-p110PITSLRE, in G2/M phase.

Figure 3: Expression of PITSLRE protein kinases during cell cycle progression in Ba/F3 cells.

(A,B) Western blot analysis with anti-PITSLRE antibodies (A) or with anti-cyclin B1 antibodies (B) of Ba/F3 cells. Cells were first synchronized in G1 by IL-3 depletion and released from this G1-block by subsequent stimulation with IL-3 for the times indicated. The corresponding cell cycle phases are indicated. (C) Northern blot analysis of total RNA preparations (30 µg) of Ba/F3 cells. The RNA samples were prepared at the same time points as the samples used for Western blot analysis. A *Pst*I restriction fragment (1072 bp) of the mouse p110^{PITSLRE} cDNA was used as a probe. Numbers to the left indicate the length of RNA markers (kb).

Figure 4: Identification and mapping of an IRES element in the coding region of PITSLRE mRNA.

(A) Schematic representation of a dicistronic mRNA and of different p110^{PITSLRE}-specific sequence elements that were cloned as an ICS between the coding regions for LUC and LACZ. Nucleotide numbers indicate the positions based on the p110^{PITSLRE} cDNA. (B, C) The dicistronic plasmids (Di-1 - Di-6) depicted in panel A and the dicistronic plasmid Δ ppt were transiently transfected in 293T cells and expression of LUC and LACZ was analyzed by measurement of their enzymatic activity (bars are representative for four independent transfections) and by Western blot analysis. In the latter case 293T cells transfected with pSV-Sport-LUC (LUC) or pSV-Sport-LACZ (LACZ) served as positive control. The upper and the lower panels show detection with anti-luciferase and anti- β -galactosidase antibodies, respectively. (D) Comparison of LUC and LACZ expression in 293T cells transiently transfected with Di-1 or HPDi-1 which carries an additional hairpin downstream from the SV40 early promoter. (E) Northern blot analysis of dicistronic mRNA expression in 293T cells transfected with the dicistronic constructs indicated on top of each lane (5 µg total RNA/lane), as

17

revealed with LUC-(left) and LACZ-(right) specific probes. Detection of LUC and LACZ induced by overexpression of pSV-Sport-LUC and pSV-Sport-LACZ served as a positive control.

- 5 **Figure 5: Upregulation of internal ribosome entry on dicistronic mRNA Di-1 in G2/M-specific Ba/F3 cells.**
Ba/F3 cells were stably transfected with the dicistronic expression plasmid Di-1, and expression of LUC (upper panel) and LACZ (lower panel) was analyzed in two representative non-synchronized clones. (B) Four different Ba/F3-Di-1 clones
10 were synchronized in G1 (open bars) and G2/M (filled bars) by IL-3-depletion (14 h) and restimulation (24 h) with IL-3, respectively. Specific activity of LACZ and LUC was analyzed and expressed as the ratio between LACZ and LUC activities. Data are the mean \pm s.d. of triplicates.

15

The current invention is further described and explained by way of the following non-limiting examples. A section disclosing the materials and methods used in the Examples is included too.

EXAMPLES

20

Example 1

The internal initiation of translation on the PITSLRE p110 (α 2-2) mRNA is mediated by an IRES element present in the coding region.

- 25 A possible mechanism accounting for the synthesis of p58 is leaky ribosome scanning (Kozak, 1989, 1991). According to this model proposed by Kozak and coworkers, the small subunit of the ribosome first recognizes the 5' terminal cap structure of an mRNA and then scans the mRNA sequence in a 5' to 3' direction for potential AUG initiation codons. Often, but not always, the first AUG is

utilized. Whether this AUG is selected or ignored depends largely on the sequence context surrounding it. An optimal nine nucleotide consensus sequence, 5'-CC(A/G)CCAUGG-3', has been derived on the basis of extensive mutagenesis experiments (Kozak, 1986). The presence of a purine in position -3 is most important for efficient AUG usage. In the absence of a -3 purine, the presence of a guanosine at position +4 is essential. Ribosomal subunits that fail to initiate at the first AUG can continue their search for an AUG in a more favorable sequence context.

Inspection of the PITSLRE mRNA sequence reveals a poor to moderate match around the first AUG and a very poor match for the nucleotides flanking the AUG of p58 (table 1). If the scanning mechanism would be used for translational initiation of p58, the ribosomal 43S ternary complex would bind at the 5' end of the mRNA and would scan 1011 nucleotides, bypassing seventeen AUG codons to initiate protein synthesis at the eighteenth AUG codon. Several of the upstream AUG codons are in a more favourable context to initiate protein synthesis than the eighteenth AUG (table 1). Because the observations according to the current invention are not compatible with the leaky scanning model, the possibility of the presence of an IRES sequence in the coding region of the PITSLRE mRNA was examined. Bicistronic mRNAs have been effectively used *in vivo* to demonstrate the existence of IRES sequences in both viral and cellular mRNAs. A plasmid vector in which the SV40 promotor drives the transcription of a capped bicistronic transcript was constructed in the present invention (fig. 4A). The first cistron, encoding luciferase (Luc) should be translated by the conventional cap-dependent scanning mechanism. However, as ribosomes fail to continue scanning through the intercistronic spacer (ICS) insert, the second cistron, encoding β -galactosidase (LacZ), should be translated only if the preceding sequence contains an IRES. The region starting from position 121 was first subcloned and ending up at the internal initiation codon ATG (p58) (position 1126) by PCR amplification (di-1, fig. 4 A) and the plasmid was transiently transfected into the 293 T cell line. The

translation products were monitored in enzymatic assays (fig. 4B) and by western blotting (figure 4C). As expected, the dicistronic mRNA produced luciferase. Interestingly, the same lysate was also positive in the LacZ activity test. Western blot analysis showed that both translation products were of the correct size
5 excluding the occurrence of fusion proteins (fig. 4C). The observation that LacZ is translated from the dicistronic transcript suggests the presence of an internal ribosomal entry site in the ICS.

To exclude the possibility that the function of the potential IRES-element is to promote the transfer of initiation-competent ribosomes from the termination
10 codon of the upstream cistron to the initiation codon of the downstream cistron, a hairpin near the 5' end was inserted (HPDi-1). Figure 4D shows that this modification only negatively influences translation of the first cistron while LacZ expression remains unaffected. If enhanced ribosomal read through is responsible for the ICS responsible stimulation of LacZ then this activity should be reduced by
15 an equivalent amount.

In addition two constructs were made in which the p58 coding region in p110 PITSLRE in frame was exchanged by the coding region of luciferase. In one of them a frame shift was induced in the PITSLRE specific region by deleting
20 two nucleotides (926/927). Both cDNAs were transiently transfected into 293T cells. The respective translation products of the in frame fusion mRNA and the frame shift fusion mRNA were analysed by immunoblotting with anti-Luc antibody. In accordance with p110PITSLRE, in the cell lysates of the in frame fusion mRNA two translation products were detected: a fusion product of 130 kDa (p110-IUC) and luciferase. In the cell lysates of the frame shift
25 fusion mRNA only one translation product, luciferase, was detected. The frame shift abrogated the translation of the fusion protein of 130 kDa, while internal translation of luciferase conferred by the PITSLRE IRES element remained unaffected.

To exclude the possibility that LacZ was expressed from a monocistronic mRNA that might have been generated if the IRES element had sites for cleavage by a specific ribonuclease or if the IRES had a cryptic promotor element, Northern blot analysis was performed. The same mRNA was detected both by luciferase and LacZ probes. This observation demonstrated that both cistrons were translated from an intact dicistronic form of the mRNA (fig. 4E).

To determine whether the cellular environment in the G2/M stage of the cell cycle is more supportive for internal initiation of translation mediated by the PITSLRE IRES element, the dicistronic vector was stably transfected into the Ba/F3 cell line. Several clones were obtained and analysed during cell cycle progression (FIG. 5). Luciferase and LacZ activity were determined in cell lysates prepared at different time points during cell cycle progression. The G2/M stage is associated with enhanced LacZ activity, corrected for the amount of mRNA by measurement of luciferase activity (fig. 5B). A 3-5 times enhanced LacZ/Luc ratio was measured in the G2/M stage compared to the G1. Hence, it seems that the G2/M-stage facilitates the internal initiation.

The fact that LacZ activity can still be detected at the G1 stage of the cell cycle might be a consequence of the higher stability of the protein compared to the stability of p58 (fig. 5A).

20

Table 1

N° AUG	AUG position	AUG context (CC(A/G)CCAUGG)
1	112 (p110)	CUCAAAUGG
2	119	GGGUGAUGA
3	152	UUUAGAUGA
4	227	UUCUGAUGA
5	283* (p105)	ACUGCAUGG
6	328*	ACUCUAUGG

7	350	AGAAGAUGA
8	382*	AGCAAAUGU
9	416	AAAAGAUGA
10	440	AAAGCAUGC
11	519	CGGGAAUGG
12	544*	GGGAAAUGG
13	578	GGGGAAUGA
14	581	GAAUGAUGG
15	646*	GCAAGAUGC
16	757*	GAACGAUGA
17	874*	AGAAAAUGG
18	1126* (p58)	AAGAAAUGA

*: in frame AUG

in bold : matches with consensus sequence according to Kozak, 1986.

5 Example 2

Characterization of the PITSLRE IRES element

- The cloned fragment in the ICS in the dicistronic vector, di-1, contains 1005 nucleotides. Regions described to contain IRES activity are maximal 450 bp long.
- 10 To map the region with IRES activity in the PITSLRE mRNA a series of dicistronic plasmids was generated containing decreasing lengths of sequence coding for p110PITSLRE. Different fragments (fig. 4) were inserted into the intercistronic spacer region (di 2 to di 6) and transient transfected dicistronic plasmids were introduced into the 293T cell line. As expected, all dicistronic
- 15 mRNAs produced luciferase. The luciferase activity was used as an internal control for the different transfection efficiencies of the plasmids.

The ability of the truncated sequences to promote internal ribosomal entry on the dicistronic mRNA was compared to di-1.

The constructs containing fragments of the 5' end of the analysed region did not score in the LacZ activity test (fig. 4B, di-2, di-3). Extended deletions at the 3' end completely abrogated internal initiation, suggesting that the IRES element is situated upstream of the internal initiation codon.

Interestingly, deletion of 347 nts at the 5' end had little effect on the activity of the downstream cistron (di-4) although it resulted in a LacZ product that is slightly bigger (fig. 4C). Larger deletions of 624 nts (di-5) and 786 nts (di-6) resulted in a corresponding reduction of internal ribosome entry of 25% and 50%, respectively. This partial loss of IRES activity may reflect loss of secondary or tertiary structure elements that confer positive or negative effects or loss of protein-binding sites. The IRES activity harbored in the ICS of di-4 is comparable in strength with the activity in the ICS of di-1. The fact that it is possible to make a small deletion at the 3' end without a dramatic loss of activity has also been described for the *c-myc* IRES (Stoneley *et al.*, 1998).

Analysis of the translation products from the different dicistronic mRNAs by Western blot analysis, shown in figure 4C, shows that di-1, di-5 and di-6 express LacZ of the expected size. Di-4, which also scored positive in the LacZ activity test, expresses a larger LacZ specific translation product. It seems that deletion of a small region immediately upstream of the AUG of internal initiation of translation does not interfere activity, in casu ribosome binding, but affects the site of initiation of translation taken by the ribosomes. In various viral IRESes 3' end deletions located within the IRES complete ablate ribosomal entry.

The PITSLRE IRES element contains a purine-rich tract (93 % A/G) of 90-nucleotides which is situated 60-nucleotides upstream of the AUG (p58). A similar polypurine motif has also been found in a tobamoviral IRES (Ivanov *et al.*, 1997). The functional significance of this motif is still unclear. Possibly, it plays a functional role in analogy with the oligopyrimidine motif that has been described

23

for the picornavirus IRES (Pilipenko *et al.*, 1992; Jackson *et al.*, 1994).

The cloned fragment in the ICS in the dicistronic vector, Δ ppt, is similar to the corresponding region in Di-1, but contains a deletion of the purine-rich tract starting at position 978 and ending up at position 1065.

5 In figure 4B, we show that this deletion ablates IRES mediated internal initiation.

Example 3

Structural features of the PITSLRE IRES element

10 The importance of RNA secondary and tertiary structure for IRES function emerges from a comparison of the sequences and secondary structures of different IRESes. A common RNA structural motif involved in the internal initiation of cellular mRNAs has been proposed by Le and coworkers (Le and Maizel, 1997). A common RNA structural motif, including a Y-type stem-loop followed by a stem-
15 loop is a conserved property found in cellular IRES elements. One remarkable property of the stem-loop is that this structure is situated just upstream from the authentic initiator (Le and Maizel, 1997). The secondary structure of a fragment of 491 bp (nt 637 to 1128) of the p110PITSLRE mRNA is shown to contain IRES activity. This secondary structure is predicted by the Zuker procedure (computer
20 program: mfold).

Two structural domains seem to correlate with IRES activity. A Y-type stem-loop (689-823) and stem-loop (1069-1105). $\Delta G = -128.2$ kcal/mol.

MATERIAL AND METHODS

Plasmid constructions

- The p110^{PITSLRE} cDNA was obtained by reverse transcription and polymerase chain reaction (PCR) amplification of total mRNA from human HL-60 cells using Superscript reverse transcriptase (Life Technologies, Inc.) and High Fidelity DNA polymerase (Boehringer). The 5'- and 3'-primers used for this amplification, 5'-TGACCGGAATTCATGGGTGATGAAAAGGACTCTTGG-3' and 5'-TGACCGGAATTCTGACCTTCAGAACTTGAGGCTGAAGCC-3', respectively, gave a cDNA fragment of 2400 bp. The PCR fragment was digested with the restriction enzyme *EcoRI* and cloned into the pMA58 plasmid. This construction was used to perform site directed mutagenesis by a chloramphenicol-selection procedure using a commercially available kit (Transformer, Clontech). Briefly, this method involves simultaneously annealing of two oligonucleotide primers to one strand of the denatured double-stranded plasmid (pMA-p110^{PITSLRE}). One primer introduces the desired mutation. The second primer induces a gain of function mutation in the gene encoding chloramphenicol resistance for the purpose of selection.
- To fuse an E-tag at the 3'-end of p110^{PITSLRE} cDNA an in frame *NotI* restriction site was introduced at the stop codon by using the mutation primer 5'-AGCCTCAAGTTCGCGGCCGCAGAGTGGACC-3'. As an *EcoRI/NotI* fragment the p110^{PITSLRE} cDNA was inserted in the *EcoRI/NotI* opened pSV-Sport-E-tag plasmid. The latter was obtained by insertion of the E-tag as a *NotI/XbaI* fragment in the *NotI/XbaI* opened pSV-Sport plasmid.
- The following primers were used for the mutation of the internal initiation codon and for the induction of the frame shift respectively: 5'-GAGGAAGAAGCGAGTGAAGAT-3' and 5'-GACAGCGAGAAAGACCAGCTCG-3'.

25

The dicistronic vectors were made by first cloning the PCR-fragments and LacZ gene into the pUC19 plasmid performed by a three points ligation. In a subsequent three points ligation the PCR-fragment fused to LacZ gene was inserted together with the firefly luciferase gene into the pSV-Sport plasmid.

- 5 The 5'-end - and 3'-end primers used for amplification of the different fragments cloned into the intercistronic spacers of the different dicistronic vectors: di-1: sense: 5'CTAGTCTAGAAAAGTGAAAACCTTTAGATGAAATTC-3'; antisense: 5'TTCTTCATCTTCACCCATGGCTTCCTCACTTAC3'; di-2: sense: idem di-1; antisense: 5' TGCATGCCATGGTCCTCTCTCATCGTTCGGTGATG 3'. di-3: sense: idem di-1; antisense: 5' TGCATGCCATGGATGTCGTTTCCGACGTTTCGTGC G3'. di-4: sense: 5' CTAGTCTAGAGCACGAACGTCGGAAACGACA G3'; antisense: 5'CATGCCATGGTCTTCCTCTCGCTGTGCTGATGTC3'. di5: sense: 5'CTAGTCTAGACATCACCGAACGATGAGAGAGG3'; antisense: idem di-1. di6: sense: 5'CTAGTCTAGAGACATCAGCGACAGCGAGAGGAAGACCAGC-3'; antisense: idem di-1. The PITSLRE specific ICS in Δ ppt was obtained by PCR amplification. Two fragments were amplified with following primers: 5'-CTAGTCTAGAAAAGTGAAAACCTTTAGATGAAATTC-3'; 5'-CCATCGATAGAACCTGAGCCTGATTCTGCTGACGA-3' and 5'-CCATCGATAACCGGCAGCAACTCTGAGGAGGCATC-3'; 5'-TTCTTCATCTTCACCCATGGCTTCCTCACTTAC-3'.

- 20 The subsequent PCR fragments were digested with the restriction enzymes: *XbaI* and *NcoI*. These fragments were cloned together with LacZ gene as a *NcoI/SalI* fragment (from pIRES-lacZ) in a *XbaI/SalI* opened pUC19 plasmid. For the Δ ppt, the fragments obtained were digested with *XbaI/ClaI* and *ClaI/NcoI* respectively, and ligated with a *NcoI/SalI* LacZ containing fragment in *XbaI/SalI* opened pUC19. Subsequently, the complete insert was cloned as an *XbaI/SalI* fragment in the *KpnI/SalI* opened pSV-Sport plasmid together with the firefly luciferase gene that was cloned as an *KpnI/XbaI* fragment (from the pGL3-basic vector (Clonotech).

26

5 A stable hairpin ($\Delta G = -40$ kcal/mol) was created by introduction of a double-stranded oligonucleotide (5'CGCGTGGCGAGATTTTCAGGAGTCAC3' and 5'TCGAGTGACTCCTGAAAATCTCGCCA3') between the *MluI* and *XhoI* sites of vector pGL3-basic upstream of the luciferase gene. This was accomplished by ligation of the double-stranded oligonucleotide (with *MluI* and *XhoI* ends) with the *MluI/XhoI*-opened pGL3-basic vector.

Cells and DNA transfection

10 Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat inactivated FCS. The IL-3-dependent mouse pro-B cell line Ba/F3 (Palacios and Steinmetz, 1985) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat inactivated FCS and 20% (v/v) conditioned medium from the WEHI-3B cells as a source of mIL-3.

15 293T cells were transiently transfected by a calcium phosphate precipitation method (O'Makoney and Adams, 1994). Cells were incubated for at least 4 h with the transfection solution followed by adding fresh medium. Cells were collected by centrifugation at 48 post-transfection and were further analysed.

20 Ba/F3 cells were stably transfected by electroporation. Before transfection, cells were collected and resuspended at 1×10^7 cells per ml in medium. 20 μ g of p110PITSLRE plasmid and 5 μ g carrying a puromycine resistance gene (pBSpacdeltap) (De la Luna, S. et al., 1988) was added to 0.8 ml of cell suspension. Electroporation was performed using the Easy Ject apparatus (Eurogentec) at 1500 μ F and 300 V. Subsequently, the cells were resuspended in

25 WEHI-3B supernatant supplemented growth medium. Selection was initiated 48 h after transfection in medium containing 1 mg/ml puromycin (Sigma). After one week, surviving cells were cloned by limiting dilution. Positive transfectants were selected on the basis of immunoblotting or reporter gene expression measured by enzymatic assays.

Western blot analysis

For Western blot analysis cells were lysed in a 1% NP40 lyse buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10 % glycerol, 1 mM Pefablock (Merck), 200 u/ml aprotinin, 10 mM EDTA, 10 µg/ml leupeptin).

- 5 Total proteins were quantified in the precleared cell lysates by the Biorad assay (A295) and 50 µg of proteins were subjected to SDS-PAGE and transferred by electroblotting onto a nitrocellulose membrane. E-tag fused proteins were immunodetected with the mouse monoclonal anti-E-tag antibody (1/1000 dilution) (Pharmacia). LacZ and firefly luciferase were immunodetected with the mouse
10 monoclonal anti LacZ (1/1000 dilution) (Boehringer) and rabbit polyclonal anti-luciferase antibody (1/2000 dilution) (Promega), respectively.
PITSLRE protein kinases and Cyclin B1 were immunodetected by the rabbit polyclonal anti PITSLRE antibody (1/1000) (Santa Cruz) and the rabbit polyclonal anti cyclin B1 antibody (dilution 1/1000) (Santa Cruz), respectively.
15 Antibodies were detected with an enhance chemiluminescence kit (Amersham).

Reporter gene assays

For the reporter gene assays, cells were lysed in 25 mM tris phosphate pH 8, 2 mM DTT, 2 mM CDTA, 10 % glycerol, 1% triton X-100.

- 20 Firefly luciferase was assayed in a volume of 30 µl. The reactions were initiated by addition of 15 µl of luciferase assay/substrate buffer (40 mM Tricine, 2 mM (MgCO₃)4Mg(OH)₂.H₂O, 5 mM MgSO₄, 66 mM DTT, 0.2 mM EDTA, 0.5 mM CoA, 1 mM ATP, 1mM D-luciferin) to 15 µl cell lysate. The light signal was measured using a Top-Count (Packard).
- 25 β-galactosidase was measured in a volume of 200 µl. Twenty µl lysate was added to 160 µl substrate buffer (60 mM Na₂HPO₄, 10 mM KCl, 1 mM β-ME) reaction was initiated by adding 20 µl of 50 mM chlorophenolred-β-D galactopyranoside (CPRG). The colorimetric signal was measured at 595 nm.

28

Cellular RNA purification and Northern blotting

Total RNA was isolated making use of the RNAeasy kit (Quiagen) according to manufacturer's instructions.

- 5 Total cellular RNA (10 µg/lane) was denatured in formaldehyde and electrophoresed through a 1.2 % formaldehyde-agarose gel. RNAs were transferred onto a nylon membrane (Amersham) by the capillary blot procedure. The filters were UV cross-linked using a UV Stratalinker apparatus (Stratagene) and were hybridized with the indicated cDNAs labeled with ³²P by randomly primed DNA
- 10 synthesis. The hybridization probes were Luciferase (a 1700 bp cDNA *NcoI/XbaI* restriction fragment), LacZ (a 800 bp cDNA *Nco I/ClaI* restriction fragment), PITSLRE (a 650 bp *PvuII* restriction fragment) and an E-tag probe (5'-ACGCGGTTCCAGCGGATCCGGATACGGCTCCGGCGCACCT-3').

15

20

25

30

REFERENCES

- Beyaert, R., Kidd, V.J., Cornelis, S., Van de Craen, M., Denecker, G., Lahti, J.M., Gururajan, R., Vandenabeele, P. and Fiers, W. (1997). Cleavage of PITSRE
5 kinases by ICE/CASP-1 and CPP32/CASP-3 during apoptosis induced by tumour
necrosis factor. *J. Biol. Chem.* 272, 11694-11697.
- Bunnell, B.A., Heath, L.S., Adams, D.E., Lahti, J.M. and Kidd, V.J. (1990).
Increased expression of a 58-kDa protein kinase leads to changes in the CHO cell
cycle. *Proc. Natl. Acad. USA*, 87, 7467-7471.
- 10 De la Luna, S., Inmaculada, S., Pulido, D., Ortin, J., and Jimenez, A.
(1988). Efficient transformation of mammalian cells with constructs containing a
puromycin-resistance marker. *Gene* 62, 121.
- Eipers, P.G., Barnoski, B.L., Han, J., Carroll, A.J. and Kidd, V.J. (1991).
Localization of the expressed p58 protein kinase chromosomal gene to
15 chromosome 1p36 and a highly related sequence to chromosome 15. *Genomics* 11,
621-629.
- Gerard, R.D., Collen, D. (1997). Adenovirus gene therapy for
hypercholesterolemia, thrombosis and restenosis. *Cardiovasc. Res.* 35:451-8.
- Ivanov, P.A., Karpova, O.V., Skulachev, M.V., Tomashevskaya, O.L.,
20 Rodionova, N.P., Dorokhov, Y.L. and Atabekov, J.G. (1997). A tobamovirus
genome that contains an internal ribosome entry site functional in vitro. *Virology*
232, 32-43.
- Jang SK, Krausslich HG, Nicklin MJ, Duke GM, Palmenberg AC, Wimmer
E (1988). A segment of the 5' nontranslated region of encephalomyocarditis virus
25 RNA directs internal entry of ribosomes during in vitro translation. *J Virol*
62(8):2636-43.
- Jackson, R.J., Hunt, S.L., Gibbs, C.L. and Kaminski, A. (1994). Internal
initiation of picornavirus RNAs. *Mol. Biol. Rep* 19, 147-159.

Kozak, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* 44, 283-292.

5 Kozak, M., (1989). The scanning model for translation: An update. *J. Cell. Biol.* 108, 229-241.

Kozak, M. (1991). An analysis of vertebrate mRNA sequences: Intimations of translational control. *J. Cell. Biol.* 115, 887-903.

Lahti, J.M., Valentine, M., Xiang, J., Jones, B., Amann, J., Grenet, J., Richmond, A., Look, T. and Kidd, V.J. (1994). Alterations in the PITSLRE protein kinase gene complex on chromosome 1p36 in childhood neuroblastoma. *Nat*
10 *Genet.* 7, 370-375.

Lahti, J.M., Xiang, J., Heath, L.S., Campana, D. and Kidd, V.J. (1995). PITSLRE protein kinase activity is associated with apoptosis. *Mol. Cell. Biol.* 15, 1-11.

15 Le, S-Y and Maizel, J.V. (1997). A common RNA structural motif involved in the internal initiation of translation of cellular mRNAs. *NAR*, 25, 362-369.

Loyer, P., Trembley, J., Lahti, J.M. and Kidd, V.J. (1998). The RNP protein, RNPS1, associates with specific isoforms of the p34cdc2-related PITSLRE protein kinase in vivo. *J. Cell Science* 111, 1495-1506.

20 Meyerson, M. A family of human cdc2-related kinases *EMBO J.*, 11, 2909-2917.

Molla, A., Paul, A.V. and Wimmer, E. (1991). Cell-free, de novo synthesis of poliovirus. *Science*, 254, 1647-1655.

O'Mahoney, J.V. and Adams, T.E. (1994). Optimization of experimental
25 variables influencing receptor gene expression in hepatoma cells following calcium phosphate transfection. *DNA Cell Biol.* 13, 1227-1232.

Palacios, R., and Steinmetz, M. (1985). IL-3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes in vivo. *Cell* 41, 727.

Pilipenko, E.V., Gmyl, A.P., Maslova, S.V., Svitkin, Y.V., Sinyakov, A.N. and Agol, V.I. (1992). Prokaryotic-like cis-elements in the cap-independent internal initiation of translation on picornavirus RNA. *Cell* 68, 119-131.

5 Sauer, K., Weigmann, K., Sigrist, S. and Lehner, C.F. (1996). Novel members of the cdc2-related kinase family in *Drosophila*: cdk4/6, cdk5, PFTAIRE and PITSLRE kinases. *Mol. Biol. Cell*, 7, 1759-1769.

Schwartz, R.S., Holmes, D.R. Jr., Topol, E.J. (1998). The restenosis paradigm revisited: an alternative proposal for cellular mechanisms. *J. Am. Coll. Cardiol.* 20:1284-93.

10 Stoneley, M.F., Paulin, F.E., Le Quesne, J.P., Chappell, S.A., and Willis, A.E. (1998). C-Myc 5' untranslated region contains an internal ribosome entry segment. *Oncogene* 16, 423-428.

Tang, D., Gururajan, R., and Kidd, V.J. (1998). Phosphorylation of PITSLRE p110 isoforms accompanies their processing by caspases during Fas-mediated cell-death. *J. Biol. Chem.* 273, 16601-16607.

15 Tio, R.A., Isner, J.M., Walsh, K. (1998). Gene therapy to prevent restenosis, the Boston experience. *Semin. Interv. Cardiol.* 3:205-10.

Varenne, O., Gerard, R.D., Sinnaeve, P., Gillijns, H., Collen, D., Janssens, S. (1999) Percutaneous adenoviral gene transfer into porcine coronary arteries: is catheter-based gene delivery adapted to coronary circulation? *Hum. Gene Ther.* 20 10:1105-15.

Xiang, J., Lahti, J.M., Grenet, J., Easton, J. and Kidd, V.J. (1994). Molecular cloning and expression of alternative spliced PITSLRE protein kinase isoforms. *J. Biol. Chem.* 269, 15786-15794.

VIB

32

Sequence listing

SEQ.ID.NO. 1

5

SEQ.ID.NO.2

10

15

SEQ ID.NO.3

20

25

30

35

33

5 CAGAGGAAGAAGTGGGTGAGGGAACGCCGACAGCAGCGCCCTGACAGAGGGCGACTATGT
GCCCCACTCCCCTGCCCTGTCGCCCATCGAGCTCAAGCAGGAGCTGCCCAAGTACCTGCCGGC
CCTGCAGGGCTGCCGGAGCGTCGAGGAGTTCCAGTGCCTGAACAGGATCGAGGAGGGCACCT
ATGGAGTGGTCTACAGAGCAAAAGACAAGAAAACAGATGAAATTGTGGCTCTAAAGCGGCTG
10 AAGATGGAGAAGGAGAAGGAGGGCTTCCCGATCACGTCGCTGAGGGAGATCAACACCATCCT
CAAGGCCCAGCATCCCAACATCGTCACCGTTAGAGAGATTGTGGTGGGCAGCAACATGGACA
AGATCTACATCGTGATGAACTATGTGGAGCACGACCTCAAGAGCCTGATGGAGACCATGAAA
CAGCCCTTCTGCCAGGGGAGGTGAAGACCCTGATGATCCAGCTGCTGCGTGGGGTGAAACA
CCTGCACGACAACTGGATCCTGCACCGTGACCTCAAGACGTCCAACCTGCTGCTGAGCCACGC
15 CGGCATCCTCAAGGTGGGTGACTTCGGGCTGGCGGGGAGTACGGATCCCCTCTGAAGGCCTA
CACCCCGGTCTGTTGACCTGTGGTACCGCGCCCGAGAGCTGCTGCTTGGTGCCAAGGAATA
CTCCACGGCCGTGGACATGTGGTCAGTGGGTTCATCTTCGGGGAGCTGCTGACTCAGAAGCC
TCTGTTCCCGGGAAGTCAGAAATCGATCAGATCAACAAGGTGTTCAAGGATCTGGGGACCCC
TAGTGAGAAAATCTGGCCCGGCTACAGCGAGCTCCAGCAGTCAAGAAGATGACCTTCAGCA
20 GACACCCCTACAACAACCTCCGCAAGCGCTTCGGGGCTCTGCTCTCAGACCAGGGCTTCGACC
TCATGAACAAGTTCCTGACCTACTTCCCCGGGAGGAGGATCAGCGCTGAGGACGGCCTCAAGC
ATGAGTATTTCGCGAGACCCCCCTCCCATCGACCCCTCCATGTTCCCCACGTGGCCCGCCAA
GAGCGAGCAGCAGCGTGTGAAGCGGGGCACCAAGCCCGAGGCCCCCTGAGGGAGGCCTGGGCT
ACAGCCAGCTGGGTGACGACGACCTGAAGGAGACGGGCTTCCACCTTACCACCACGAACCAG
25 GGGGCCTCTGCCGCGGGCCCCGGCTTCAGCCTCAAGTTCTGAAGGTGAGAGTGGACCCCGTCA
TGGGG

SEQ.ID.NO.4

25 GACATCAGCGACAGCGAGAGGAAGACCAGC

SEQ.ID.NO.5

30 CACGAACGTCGGAAACGACATCGAGAAGAACAGGATAAAGCTCGCCGGAATGGGAAAGACA
GAAGAGAAGGGAAATGGCAAGGGAGCATTCCAGGAGAGAAAGGGGAATGATGGCGTGTGC
CTCTTCAGGGACCGCTTGGAGCAGTTAGAAAGGAAGCGGGAGCGGGAGCGCAAGATGCGGGA
GCAGCAGAAGGAGCAGCGGGAGCAGAAGGAGCGCGAGCGGGCGGAGGAGCGGCGCAAG
GAGCGGGAGGCCCGCAGGGAAGTGTCTGCATCACCAGACGATGAGAGAGGACTACAGCGA
CAAAGTGAAAGCCAGCCACTGGAGTCGACGCCCGCTCGGCCGCCCGGGAGCGGTTTCGAGTT
GGGAGACGGCCGAAGCCAGTAAAAGAAGAGAAAATGGAAGAAAGGGACCTGCTGTCCGAC
35 TTACAGGACATCAGCGACAGCGAGAGGAAGACCAGC

SEQ.ID.NO.6

CACGAACGTCGGAAACGACATCGAGAAGAACAGGATAAAGCTCGCCGGGAATGGGAAAGACA
GAAGAGAAGGGAAATGGCAAGGGAGCATTCCAGGAGAGAAAGGGGGAATGATGGCGTGTGC
CTCTTCAGGGACCGCTTGGAGCAGTTAGAAAGGAAGCGGGAGCGGGAGCGCAAGATGCGGGA
5 GCAGCAGAAGGAGCAGCGGGAGCAGAAGGAACGCGAGCGGGCGGGAGGAGCGGCGCAAG
GAGCGGGAGGCCCCGAGGGAAGTGTCTGCACATCACCGAACGATGAGAGAGGACTACAGCGA
CAAAGTGAAAGCCAGCCACTGGAGTCGCAGCCCGCCTCGGCCCGCGGGAGCGGTTTCGAGTT
GGGAGACGGCCGGAAGCCAGTAAAGAAGAGAAAATGGAAGAAAGGGACCTGCTGTCCGAC
TTACAGGACATCAGCGACAGCGAGAGGAAGACCAGCTCGGCCGAGTCCTCGTCAGCAGAATC
10 AGGCTCAGGTTCTGAGGAAGAAGAGGAGGAGGAGGAAGAGGAGGAGGAGGAAGGGAGCACC
AGTGAAGAATCAGAGGAGGAAGAGGAAGAGGAGGAGGAGGAGACCGGCAGCAACTCTGAGG
AGGCATCAGAGCAGTCTGCCGAAGAAGTAAGTGAGGAAGAAATG

SEQ. ID. NO 7

15 GAAGAAGAGGAGGAGGAGGAAGAGGAGGAGGAGGAAGGGAGCACCAGTGAAGAATCAGAG
GAGGAAGAGGAAGAGGAGGAGGAGGAG

- SEQ.ID.NO.1 is the IRES specified sequence according to the invention
- 20 SEQ. ID. NO.2 concerns the corresponding RNA sequence of the IRES element
- SEQ.ID.NO.3 shows the nucleotide sequence of PITSLRE protein kinase (p110pitslre) (isoform α 2-2). The IRES-activity containing sequence is underlined and corresponds to SEQ.ID.NO.1.
- SEQ.ID.NO.4 is the overlapping sequence between SEQ.ID.NO.1 and
- 25 SEQ.ID.NO. 5.
- SEQ.ID.NO.5 is a sequence comprising an extension to the left end of SEQ.ID.NO.4.
- SEQ.ID.NO.6 is a sequence comprising SEQ.ID.NO.5 and SEQ.ID.NO.1 with the overlapping SEQ.ID.NO.4 included.
- 30 SEQ.ID. NO.7 is a sequence, deleted in Δ ppt

+32 9 2446610

35

Claims

1. A nucleotide sequence enabling a cell cycle dependent initiation of translation of mRNA.
- 5 2. A nucleotide sequence according to claim 1 wherein the sequence is an IRES sequence.
3. A nucleotide sequence according to claims 1 or 2 wherein the cell cycle dependency is a G2/M cell cycle dependency.
4. An isolated and/or recombinant nucleic acid molecule, preferably DNA,
10 encoding at least a functional part of an eukaryotic internal ribosomal entry site, which site in the mitotic PITSLRE protein kinase gene comprises the sequence as depicted in SEQ.ID.NO. 1 or a functional part thereof.
5. A nucleic acid molecule according to claim 4 wherein said functional part of SEQ. ID. NO. 1 comprises the sequence as depicted in SEQ. ID. NO. 7.
- 15 6. A nucleic acid molecule according to claim 4 comprising at least a part of the sequence as depicted in SEQ.ID.NO.1 or a sequence at least substantially homologous thereto.
7. A nucleic acid molecule according to claim 4 comprising at least a part of the sequence as depicted in SEQ.ID.NO.1 or a sequence which hybridizes under
20 conventional conditions to at least a part of said sequence or its complementary sequence.
8. An isolated and/or recombinant nucleic acid molecule, preferably DNA, comprising at least the sequence as depicted in SEQ.ID.NO. 4.
9. An isolated and/or recombinant nucleic acid molecule, preferably DNA,
25 comprising at least the sequence as depicted in SEQ.ID.NO. 5.
10. An isolated and/or recombinant nucleic acid molecule, preferably DNA, comprising at least the sequence as depicted in SEQ.ID.NO. 6.
11. A chimeric gene comprising the following operably linked polynucleotides:
 - a) a nucleic acid molecule according to any of the claims 1 to 10

- b) one or more control sequences.
12. A vector comprising at least a nucleic acid molecule according to any of the claims 1 to 10 or comprising a chimeric gene according to claim 11.
13. The vector according to claim 12 wherein the vector is an expression vector
5 containing at least a single promoter.
14. An eukaryotic host cell comprising a nucleic acid molecule according to any of the claims 1 to 10 or comprising a chimeric gene according to claim 11.
15. An expression system comprising an eukaryotic host cell according to claim 14.
- 10 16. A method for cap-independent translation of mRNA by including in an expression vector a translation control element or analogues thereof having the nucleic acid molecule as set forth in SEQ.ID.NO.1 and/or SEQ.ID.NO.4.
17. Use of any of the sequences of claims 1 to 10 to induce a cell cycle dependent initiation of translation in eukaryotic cells.
- 15 18. Any of the sequences of claims 1 to 10 for use in accordance with claim 17 wherein the sequence is a cell cycle dependent IRES sequence.
19. Use according to claim 18 wherein the cell cycle dependent IRES sequence is a G2/M-dependent IRES sequence.
- 20 20. Use of a vector of claims 12 or 13 or any of the sequences according to claims 1 to 10 for the preparation of a pharmaceutical composition for the treatment and/or prevention of a disease by gene therapy.

Abstract

The current invention relates to two isoforms, p110 and p58 of PITSLRE protein kinase, which can be translated from the same p110 ($\alpha 2$ -2) mRNA by an internal
5 ribosome entry process. This means that p110 and p58, two proteins with putative different functions, are translated from a single mRNA species by using two AUGs within the same reading frame. These two proteins share the 439 C-terminal amino acids that contain the kinase domain. The IRES in the polycistronic p110 mRNA is the first IRES completely localized in the coding region of a cellular mRNA.
10 Moreover, it was unexpectedly found that the IRES element is cell cycle regulated. Translation of p58 occurs in the G2/M stage of the cell cycle.

1/6

Figure 1

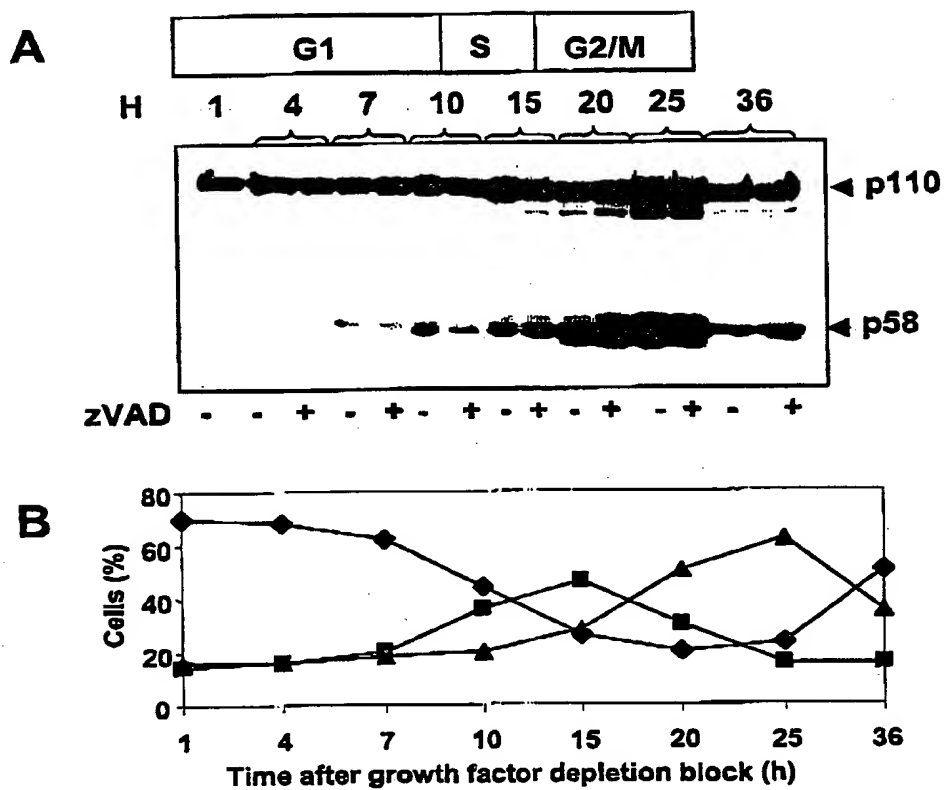
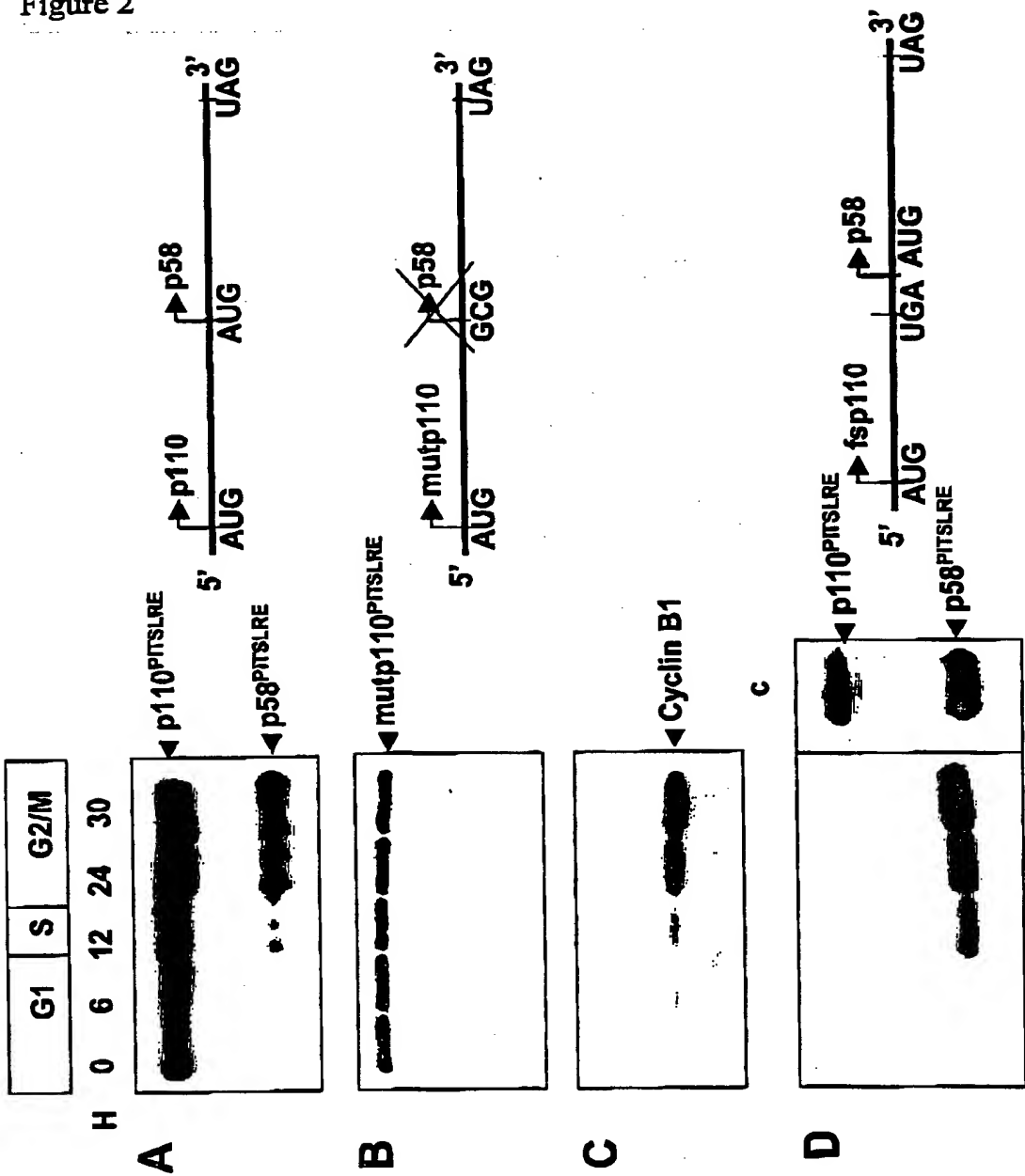


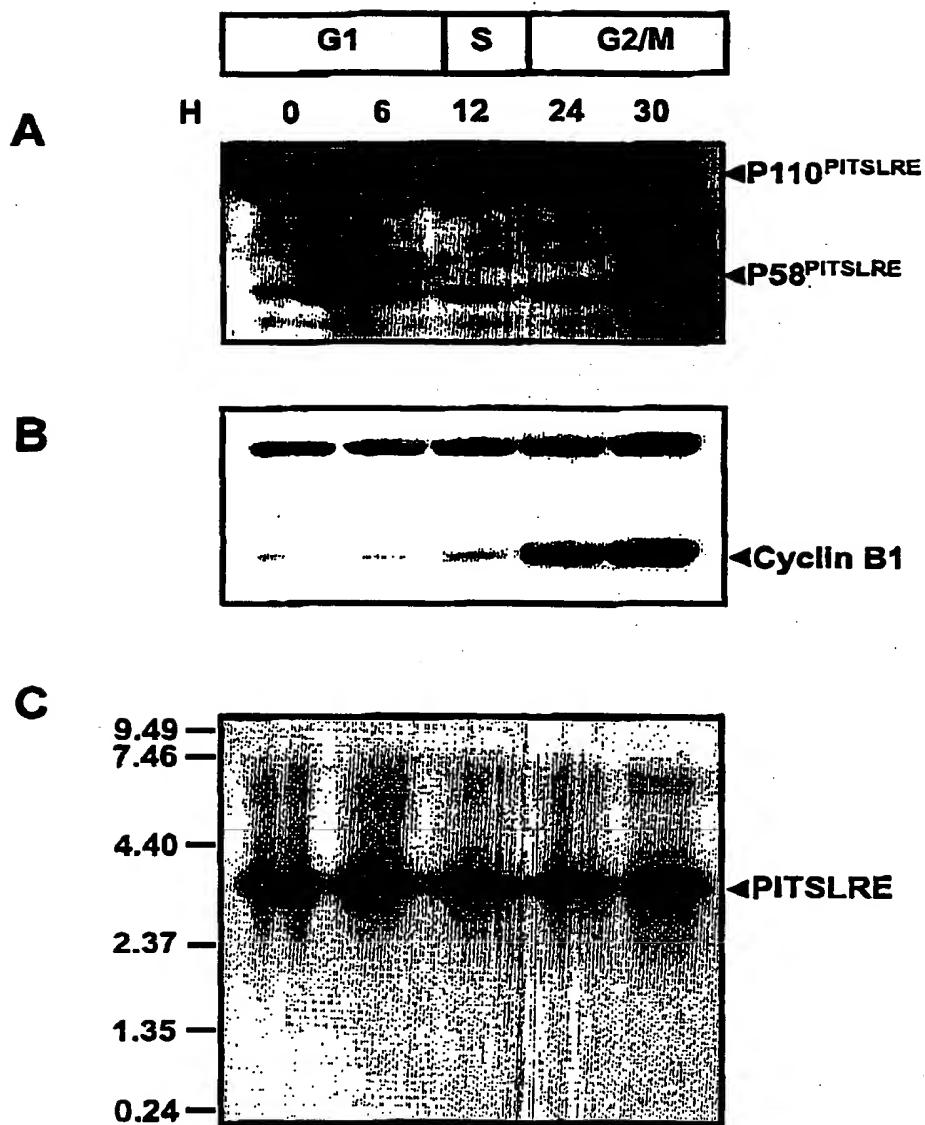
Figure 2



+32 9 2446610

3/6

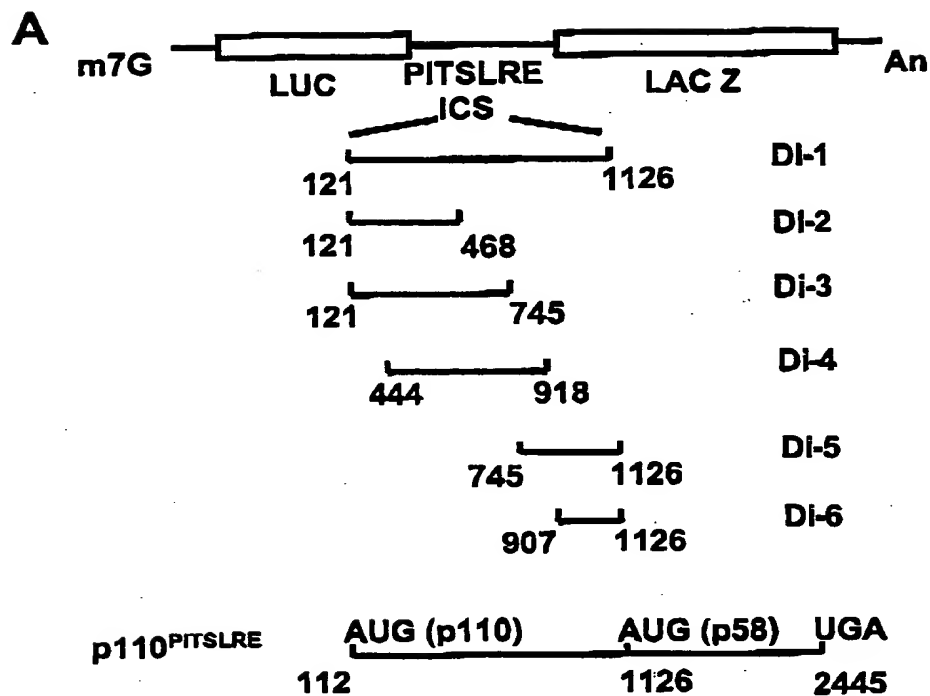
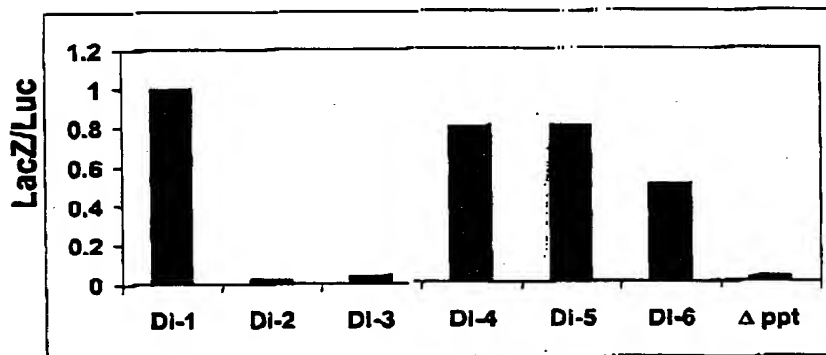
Figure 3



+32 9 2446610

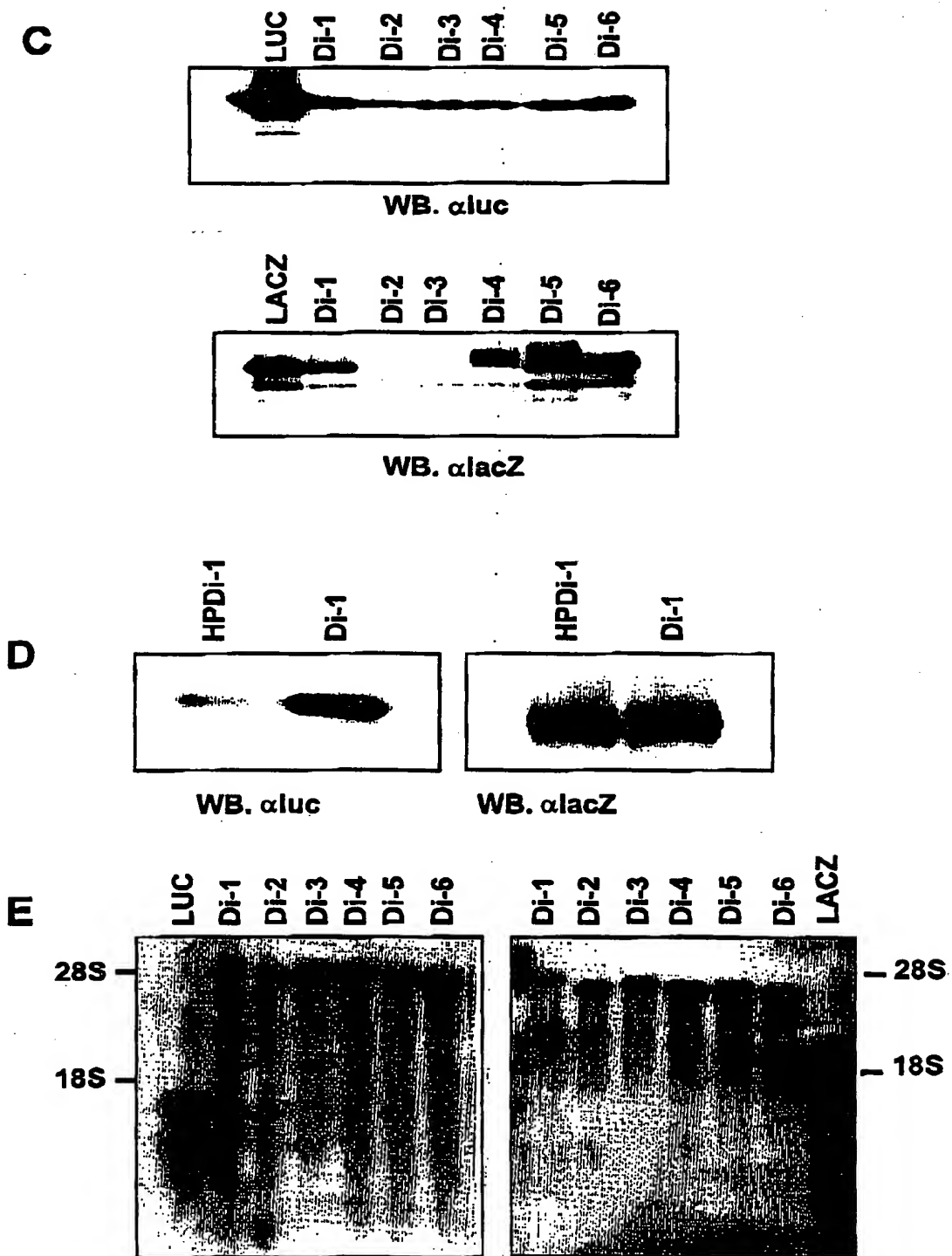
4/6

Figure 4

**B**

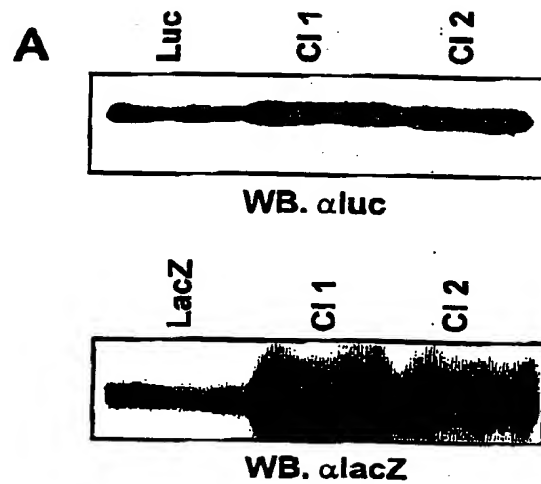
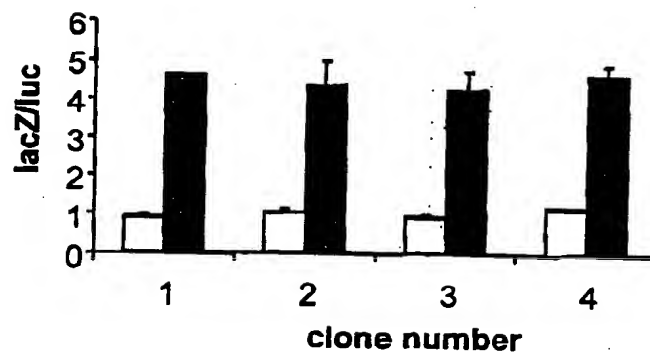
5/6

Figure 4



6/6

Figure 5

**B**

+32 9 2446610

SEQUENCE LISTING

<110> VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOL

<120> NOVEL INTERNAL RIBOSOME ENTRY SITE, VECTOR CONTAINING
SAME AND USES THEREOF

<130> V1/002-V036

<140>

<141>

<150> 99200216.2

<151> 1999-01-26

<160> 27

<170> PatentIn Ver. 2.1

<210> 1

<211> 222

<212> DNA

<213> Homo sapiens

<400> 1

gacatcagcg acagcgagag gaagaccagc tcggccgagt cctcgtcagc agaatcaggc 60
tcaggttctg aggaagaaga ggaggaggag gaagaggagg aggaggaagg gaggaccagt 120
gaagaatcag aggaggaaga ggaagaggag gaggaggaga ccggcagcaa ctctgaggag 180
gcatcagagc agtctgccga agaagtaagt gaggaagaaa tg 222

<210> 2

<211> 222

<212> RNA

<213> Homo sapiens

<400> 2

gacaucagcg acagcgagag gaagaccagc ucggccgagu ccucgucagc agaaucaggc 60
ucagguucug aggaagaaga ggaggaggag gaagaggagg aggaggaagg gaggaccagu 120
gaagaucag aggaggaaga ggaagaggag gaggaggaga ccggcagcaa cucugaggag 180
gcaucagagc agucugccga agaaguaagu gaggaagaaa ug 222

<210> 3

<211> 2471

<212> DNA

<213> Homo sapiens

+32 9 2446610

<220>

<221> misc_feature

<222> () ..)

<223> the IRES-activity containing sequence

<220>

<223> PITSLRE protein kinase (p110pitslre) (isoform alfa
2-2)

<400> 3

```
atacaggaag tgacgatact tttggcgcgcg gcggttgctg tttcttctct ggctccggga 60
ccggcgggcg cgccggcggc acggggcgcg gcgtaggggtg ttttaactca aatgggtgat 120
gaaaaggact cttggaaagt gaaaacttta gatgaaattc ttcaggaaaa gaaacgaagg 180
aaggaacaag aggagaaagc agagataaaa cgcttaaaaa attctgatga ccgggattcc 240
aagcgggatt cccttgagga gggggagctg agagatcact gcatggagat cacaataagg 300
aactccccgt atagaagaga agactctatg gaagacagag gagaagaaga tgattctttg 360
gccatcaaac caccacagca aatgtctcgg aaagaaaaag ttcatcacag aaaagatgaa 420
aagagaaaag agaaaaagca tgctagagtg aagaagaaag aaagagagca cgaacgtcgg 480
aaacgacatc gagaagaaca ggataaagct cgccgggaat gggaaagaca gaagagaagg 540
gaaatggcaa gggagcattc caggagagaa agggggaatg atggcgtgtg cctcttcagg 600
gaccgcttgg agcagttaga aaggaagcgg gagcgggagc gcaagatgcy ggagcagcag 660
aaggagcagc gggagcagaa ggagcgcgag cgccggggcg aggagcggcg caaggagcgg 720
gaggccccga gggaagtgtc tgcacatcac cgaacgatga gagaggacta cagcgacaaa 780
gtgaaagcca gccactggag tcgcagcccg cctcgcccg cgccgggagcg gttcgagttg 840
ggagacggcc ggaagccagt aaaagaagag aaaatggaag aaaggacct gctgtccgac 900
ttacaggaca tcagcgacag cgagaggaag accagctcgg ccgagtcctc gtcagcagaa 960
tcaggctcag gttctgagga agaagaggag gaggaggaag aggaggagga ggaagggagc 1020
accagtgaag aatcagagga ggaagaggaa gaggaggagg aggagaccgg cagcaactct 1080
gaggaggcat cagagcagtc tgccgaagaa gtaagtggg aagaaatgag tgaagatgaa 1140
gaacgagaaa atgaaaacca cctcttggtt gttccagagt cacggttcga ccgagattec 1200
ggggagagtg aagaagcaga ggaagaagtg ggtgagggaa cgccgcagag cagcgccctg 1260
acagagggcg actatgtgcc cgactccctt gccctgtcgc ccatcgagct caagcaggag 1320
ctgcccaagt acctgccggc cctgcagggc tgccggagcg tcgaggagtt ccagtgcctg 1380
aacaggatcg aggagggcac ctatggagt gtctacagag caaaagacaa gaaaacagat 1440
gaaattgttg ctctaaagcg gctgaagatg gagaaggaga aggagggtt cccgatcacg 1500
tcgctgaggg agatcaacac catcctcaag gcccagcatc ccaacatcgt caccgttaga 1560
gagattgttg tgggcagcaa catggacaag atctacatcg tgatgaacta tgtggagcac 1620
gacctcaaga gcctgatgga gaccatgaaa cagcccttcc tgccagggga ggtgaagacc 1680
ctgatgatcc agctgctgcg tggggtgaaa cacctgcacg acaactggat cctgcaccgt 1740
gacctcaaga cgtccaacct gctgctgagc cagcccgga tcctcaaggt ggtgaccttc 1800
gggctggcgc gggagtagcg atccccctct aaggcctaca ccccggtcgt ggtgaccttc 1860
tggtagcgcg cccagagct gctgcttggt gccaaagaa actccacggc cgtggacatg 1920
tggtagtggt gttgcatctt cggggagctg ctgactcaga agcctctgtt ccccggaag 1980
tcagaaatcg atcagatcaa caaggtgttc aaggatctgg ggacccttag tgagaaaatc 2040
tggccccggt acagcgagct cccagcagtc aagaagatga ccttcagcag acacccttac 2100
aacaacctcc gcaagcgctt cggggctctg ctctcagacc agggcttcga cctcatgaac 2160
aagttcctga cctacttccc cgggaggagg atcagcgctg aggacggcct caagcatgag 2220
tatttccgcg agacccccct ccccatcgac ccctccatgt tccccacgtg gcccgccaag 2280
```

+32 9 2446610

```

agcgagcagc agcgtgtgaa gcggggcacc agcccagaggc ccoctgaggg aggcctgggc 2340
tacagccagc tgggtgacga cgacctgaag gagacgggct tccaccttac caccacgaac 2400
cagggggcct ctgccgcggg ccccggcttc agcctcaagt tctgaaggct agagtggacc 2460
ccgtcatggg g                                     2471

```

<210> 4

<211> 30

<212> DNA

<213> Homo sapiens

<400> 4

gacatcagcg acagcgagag gaagaccagc

30

<210> 5

<211> 468

<212> DNA

<213> Homo sapiens

<400> 5

```

cacgaacgtc ggaaacgaca tcgagaagaa caggataaag ctgcccgga atgggaaaga 60
cagaagagaa gggaaatggc aaggagcat tccaggagag aaagggggaa tgatggcgtg 120
tgcctcttca gggaccgctt ggagcagtta gaaaggaagc gggagcggga gcgcaagatg 180
cgggagcagc agaaggagca gcgggagcag aaggagcgcg agcggcgggc ggaggagcgg 240
cgcaaggagc gggaggcccg cagggaagtg tctgcacatc accgaacgat gagagaggac 300
tacagcgaca aagtgaaagc cagccactgg agtcgcagcc cgctcggcc gccgcgggag 360
cggttcgagt tgggagacgg ccggaagcca gtaaaagaag agaaaatgga agaaagggac 420
ctgctgtccg acttacagga catcagcgac agcgagagga agaccagc 468

```

<210> 6

<211> 660

<212> DNA

<213> Homo sapiens

<400> 6

```

cacgaacgtc ggaaacgaca tcgagaagaa caggataaag ctgcccgga atgggaaaga 60
cagaagagaa gggaaatggc aaggagcat tccaggagag aaagggggaa tgatggcgtg 120
tgcctcttca gggaccgctt ggagcagtta gaaaggaagc gggagcggga gcgcaagatg 180
cgggagcagc agaaggagca gcgggagcag aaggagcgcg agcggcgggc ggaggagcgg 240
cgcaaggagc gggaggcccg cagggaagtg tctgcacatc accgaacgat gagagaggac 300
tacagcgaca aagtgaaagc cagccactgg agtcgcagcc cgctcggcc gccgcgggag 360
cggttcgagt tgggagacgg ccggaagcca gtaaaagaag agaaaatgga agaaagggac 420
ctgctgtccg acttacagga catcagcgac agcgagagga agaccagctc ggccgagtcc 480
tcgtcagcag aatcaggctc aggttctgag gaagaagagg aggaggagga agaggaggag 540
gaggaagggg gcaccagtga agaatacagag gaggaagagg aagaggagga ggaggagacc 600
ggcagcaact ctgaggaggc atcagagcag tctgccgaag aagtaagtga ggaagaaatg 660

```

+32 9 2446610

<210> 7

<211> 87

<212> DNA

<213> Homo sapiens

<220>

<223> Delta-ppt

<400> 7

gaagaagagg aggaggagga agaggaggag gaggaagga gcaccagtga agaatacagag 60
gaggaagagg aagaggagga ggaggag 87

<210> 8

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: 5'-end primer

<400> 8

tgaccggaat tcatgggtga tgaaaaggac tcttgg 36

<210> 9

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: 3'-end primer

<400> 9

tgaccggaat tetgaccttc agaacttgag gctgaagcc 39

<210> 10

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: mutation
primer

+32 9 2446610

<400> 10

agcctcaagt tcgcggccgc agagtggacc

30

<210> 11

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 11

gaggaagaag cgagtgaaga t

21

<210> 12

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 12

gacagcgaga aagaccagct cg

22

<210> 13

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: 5'-end primer

<400> 13

ctagtctaga aaagtgaaaa ctttagatga aattc

35

<210> 14

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: 3-end primer

+32 9 2446610

<400> 14

ttcttcacatct tcaccccatgg cttcctcact tac

33

<210> 15

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: 3'-end primer

<400> 15

tgcacgcat ggctctctct catcggttcgg tgatg

35

<210> 16

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: 3'-end primer

<400> 16

tgcacgcat ggatgtcgtt tccgacgttc gtgc

34

<210> 17

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: 5'-end primer

<400> 17

ctagtctaga gcacgaacgt cggaaacgac a

31

<210> 18

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: 3'-end primer

+32 9 2446610

<400> 18
catgccatgg tcttctctctc gctgtcgtg atgtc

35

<210> 19
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: 5'-end primer

<400> 19
ctagtctaga catcaccgaa cgatgagaga gg

32

<210> 20
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: 5'-end primer

<400> 20
ctagtctaga gacatcagcg acagcgagag gaagaccagc

40

<210> 21
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 21
ctagtctaga aaagtga aaa ctttagatga aattc

35

<210> 22
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

+32 9 2446610

<400> 22

ccatcgatag aacctgagcc tgattctgct gacga

35

<210> 23

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 23

ccatcgatac cggcagcaac tctgaggagg catc

34

<210> 24

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 24

ttcttcatct tcacccatgg cttcctcact tac

33

<210> 25

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: hairpin
forming oligonucleotide

<400> 25

cgcgtggcga gattttcagg agtcac

26

<210> 26

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: hairpin

+32 9 2446610

forming oligonucleotide

<400> 26

tcgagtgact cctgaaaatc tcgcca

26

<210> 27

<211> 40

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: E-tag probe

<400> 27

acgcggttcc agcggatccg gatacggctc cggcgcacct

40